# Activation of guanylate cyclase by *E. coli* heat-stable enterotoxin (STa)

## Modulation by NAD and pertussis toxin

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Received 15 May 1986

The heat-stable enterotoxin (STa) of *E. coli* activates intestinal guanylate cyclase and leads to increased cGMP levels by an as yet undetermined mechanism. In comparing this cGMP system to other known toxin-mediated alterations in cAMP metabolism, we observed that pertussis toxin caused lower levels of intestinal cGMP synthesis in response to purified STa. Another participant in ADP-ribosylation reactions, NAD, enhanced the ability of STa to activate guanylate cyclase, yet had no effect on basal enzyme activity. Niacinamide and isoniacinamide also had no effect on basal activity, but attenuated the STa activation. These results are discussed in relation to current models of hormone/toxin-sensitive adenylate cyclase, and may suggest an involvement of guanine-nucleotide-binding proteins in intestinal cGMP metabolism.

*Bacterial toxin* *Guanylate cyclase* *cyclic GMP* *ADP-ribosylation* *GTP-binding protein* *Secretory diarrhea*

## 1. INTRODUCTION

Enterotoxigenic strains of *E. coli* produce peptide toxins which alter intestinal water balance and lead to acute diarrheal illnesses in humans and many animals [1–3]. A large (85–90 kDa), heat-labile toxin (LT) has been well described, and shares many structural and functional homologies with cholera toxin, including the ability to ADP-ribosylate and irreversibly activate intestinal adenylate cyclase [4,5]. Another class of *E. coli* toxins are low-$M_r$, heat-stable toxins (STs) which also cause secretory diarrhea [6,7]. Within these STs, there now appear to be two types, STa and STb. STb has only been observed in pigs and calves, and has not yet been well characterized [8]. STa, on the other hand, has been purified to homogeneity, and is known to be composed of either 18 or 19 amino acids [9–11]. STa has been observed to alter dramatically cGMP metabolism via the activation of intestinal guanylate cyclase [12,13], followed by a blockade of inward ion transport and subsequent secretion of water into the intestinal lumen [14]. Exogenously added cGMP analogues are also capable of directly altering ion transport and eliciting a secretory response both in vivo and in vitro [15,16]. Thus, it is fairly evident at this point that STa alters intestinal cGMP metabolism which then leads to changes in cellular ion transport. What is less clear is the actual mechanism whereby STa activates membrane-bound guanylate cyclase in the intestine. While a number of laboratories have suggested the STa-induced liberation of calcium and/or fatty acid metabolites as a model for guanylate cyclase activation [17–20], there has been little information on how the recently described STa receptor [21,22] is actually coupled to intestinal guanylate cyclase. In wanting to study this coupling process further, we have utilized the current models of hormone-

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00145793/86/$3.50 

and toxin-sensitive adenylate cyclase to develop experimental models. Here, we present evidence that STa activation of intestinal guanylate cyclase can be modulated by NAD and pertussis toxin, and we suggest that intestinal cGMP metabolism may be influenced by guanine-nucleotide-binding proteins.

## 2. MATERIALS AND METHODS

The heat-stable enterotoxin (STa) of human *E. coli* strain 18d was purified to homogeneity using Amberlite XAD, DEAE-Sephacel and Sephadex G-25 chromatography as described [11]. Purified pertussis toxin (PT) was purchased from List Laboratories, and was activated just before use as described by Katada and Ui [23].

Intestinal villous cells were prepared from 150–200 g female Sprague-Dawley rats by the method of Gianella et al. [21]. Washed villous cells were suspended in 10 mM Tris-HCl (pH 8), 1 mM EDTA, 1 mM DTT, 0.25 M sucrose, and homogenized with 10–15 strokes in a glass Dounce homogenizer. For some experiments membranes were prepared from homogenates by centrifugation at 105000 × *g* for 60 min at 4°C. Homogenates or membranes were adjusted to a protein concentration of 5 mg/ml in homogenization buffer, and either used immediately or frozen at −70°C.

Guanylate cyclase assays were performed in 120 μl reaction mixtures containing 10–100 μg homogenate or membrane protein, 50 mM Tris-HCl (pH 7.6), 10 mM theophylline, 7.5 mM creatine phosphate, 20 μg creatine phosphokinase (200 U/mg), 1 mM GTP and 4 mM $MgCl_2$ [24]. In some experiments, STa, PT, NAD or NAD analogues were added at the noted concentrations and preincubated for 5–10 min at 37°C. Reactions were initiated by the addition of Mg-GTP and incubated for 10–30 min at 37°C. Incubations were terminated by the addition of 0.4 ml cold sodium acetate buffer (pH 4) followed by boiling for 3 min at 95°C. The amount of cGMP formed was determined by RIA [25] using a commercially obtained cGMP RIA kit (New England Nuclear, Boston). Protein was determined by the method of Lowry et al. [26]. All other reagents were of the highest quality commercially available and were obtained from Sigma (St. Louis).

## 3. RESULTS

Several laboratories have previously reported the 2–4-fold activation of intestinal guanylate cyclase by *E. coli* STa [12–14]. We also observed this activation in homogenized intestinal villous cells, and table 1 (column A) shows the effects of 0.01–1.0 μg/ml of STa on guanylate cyclase activity. We observed a small increase in guanylate cyclase activity beginning with an STa concentration of 0.05 μg/ml, and a 2.5–3-fold activation of the enzyme with saturating levels of STa (>0.5 μg/ml).

Although we and others have consistently observed this STa activation of guanylate cyclase, it is still unclear as to precisely how this activation occurs on the molecular level. In wanting to study this question further, we chose to consider the possibility that STa might share some functional homology with other bacterial toxins which alter intracellular cyclic nucleotide metabolism by well-defined mechanisms [23,27,28]. In particular, we chose PT, which is produced by *Bordetella pertussis* [29] and is known to regulate intracellular adenylate cyclase activity via ADP-ribosylation of

Table 1

Effect of *E. coli* STa on intestinal guanylate cyclase activity

| Final concentration of STa (μg/ml) | Guanylate cyclase activity (pmol cGMP/mg per 10 min) | |
|---|---|---|
| | A | B |
| 0 | 11.3 ± 0.2 | 13.5 ± 0.3 |
| 0.01 | 11.6 ± 0.1 | 22.6 ± 0.6 |
| 0.05 | 18.7 ± 0.5 | 41.3 ± 0.3 |
| 0.10 | 20.6 ± 0.2 | 59.4 ± 0.6 |
| 0.50 | 27.7 ± 0.2 | 71.1 ± 1.8 |
| 1.0 | 27.7 ± 0.4 | 70.0 ± 3.4 |

The indicated concentrations of purified *E. coli* STa were added to reaction tubes containing 50 μg villous cell homogenate protein and all other components of the guanylate cyclase assay. Reactions were initiated by the addition of Mg-GTP substrate, and analyzed for cGMP formed by RIA (see section 2). Column B shows the results when 1 mM NAD was added for 5 min (37°C) before the addition of STa and Mg-GTP. Each point represents data of at least duplicate determinations, and representative of 3 or more experiments

a specific guanine-nucleotide-binding protein, $G_i$ [23,30].

Fig.1 shows that the addition of up to 10 $\mu$g purified PT had no effect on basal guanylate cyclase, but surprisingly, led to a 50% decrease in the STa activation of the enzyme. This PT effect was dose-dependent and was observed in a large number of experiments in which the PT blockade of STa activation varied from 50 to 75% depending on the villous cell preparation and the quantity of PT used (not shown). Freshly prepared homogenate and membranes were generally more sensitive to PT than were frozen preparations.

Since PT is known to function enzymatically as an ADP-ribosyl transferase [23,30], it seemed logical to add the other substrate for the ADP-ribosylation reaction, NAD, and see if this had any additional effect on intestinal guanylate cyclase. Fig.2 shows that NAD had no effect on basal activity at any of the concentrations tested, while similar amounts of NAD led to marked enhancement of the STa activation. The NAD effect was dose-dependent, and led to 6–10-fold increases in guanylate cyclase as opposed to the 2–4-fold increases seen in the absence of NAD. Also, as shown in table 1 (column B), the presence of 1 mM

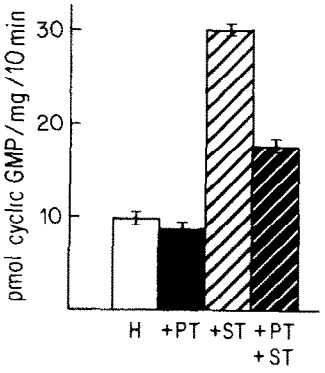


Fig.1. Effect of pertussis toxin on STa activation of intestinal guanylate cyclase. 50 $\mu$g villous cell homogenate protein was combined with all components of the guanylate cyclase assay except for Mg-GTP substrate. Assay tubes then received either 10 $\mu$g activated PT or an equivalent volume (10 $\mu$l) of buffer, and were preincubated for 10 min at 37°C. Mg-GTP was then added to initiate the reaction and the guanylate cyclase assay was performed and analyzed as described in section 2. Each point represents data of at least duplicate determinations, and is representative of 3 or more experiments.

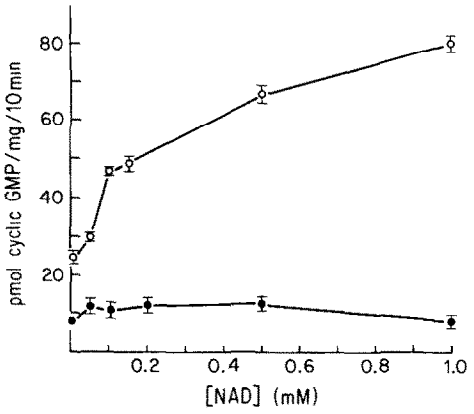


Fig.2. Effect of NAD on basal and STa-activated guanylate cyclase. The indicated concentrations of NAD were added to reaction tubes containing membrane protein and all other components of the guanylate cyclase assay (see section 2 and fig.1). Assays were performed in the absence (●—●) or presence (○—○) of 100 ng STa, and were initiated by the addition of Mg-GTP substrate.

NAD allowed us to demonstrate activation of guanylate cyclase with considerably less STa than was required in the absence of NAD. Fig.3 shows an almost 10-fold activation of intestinal guanylate cyclase in response to STa in the presence of 1 mM NAD, and in other experiments, the addition of 10 $\mu$g PT also caused a 50% inhibition of the NAD enhanced STa effect (not shown).

In other experiments, we also found that the NAD enhancement of STa activation persisted in the presence of equimolar amounts of NADH, suggesting that the NAD effect was not simply due to a change in pyridine nucleotide redox. Moreover, the data suggested to us that an NAD driven ADP-ribosylation of a cellular protein might be modulating the STa effect on cGMP metabolism. To test further this idea, we utilized niacinamide and isoniacinamide, two known competitive inhibitors of ADP-ribosylation reactions [31]. Table 2 shows that neither reagent altered basal guanylate cyclase, but both were capable of markedly decreasing the STa activation of the enzyme indicating an NAD-sensitive step in the process.

## 4. DISCUSSION

ADP-ribosylations of membrane-bound regula-

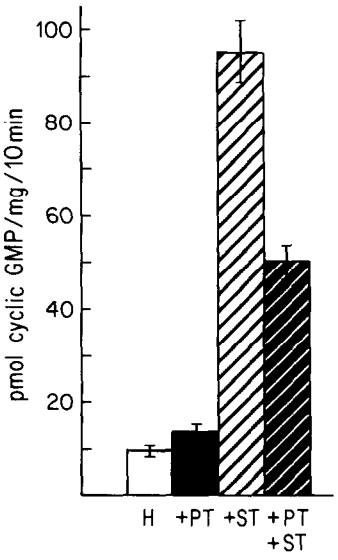


Fig.3. Effect of pertussis toxin on STa activation of intestinal guanylate cyclase in the presence of 1 mM NAD. This experiment was performed as described in fig.1, with the exception that 1 mM NAD was added to all reaction tubes before initiating the reaction with Mg-GTP as described in section 2.

Table 2

Effect of niacinamide and isoniacinamide on STa activation of guanylate cyclase

| Addition | Guanylate cyclase activity (pmol cGMP/mg per 10 min) | |
|---|---|---|
| | – NAD | + 1 mM NAD |
| None | 13.0 ± 0.4 | 11.8 ± 0.2 |
| 10 mM niacinamide | 11.6 ± 0.5 | 10.0 ± 0.5 |
| 10 mM isoniacinamide | 11.7 ± 0.2 | 10.5 ± 0.4 |
| STa (1 μg/ml) | 30.0 ± 1.5 | 111.5 ± 6.1 |
| STa plus 10 mM niacinamide | 21.6 ± 0.35 | 80.7 ± 4.1 |
| STa plus 10 mM isoniacinamide | 21.5 ± 0.2 | 68.0 ± 5.0 |

Homogenates were prepared from isolated villous cells and assayed for guanylate cyclase activity in the presence of the noted additions. Reaction conditions and cGMP determinations were as described in section 2

tory proteins are now well documented mechanisms whereby other bacterial toxins, such as those of *Vibrio cholerae* and *B. pertussis* are able to alter intracellular cAMP levels in infected cells [4,5,23]. Both cholera and pertussis toxins are known to regulate adenylate cyclase at the level of the guanine-nucleotide-binding proteins, $G_s$ and $G_i$, which serve as coupling factors between several membrane receptors and the catalytic subunit of adenylate cyclase [30]. Our current observations on the ability of both NAD and PT to modulate the STa activation of guanylate cyclase might also suggest the involvement of $G_i$ or a related guanine-nucleotide-binding protein in intestinal cGMP metabolism. Such an involvement of G-proteins with cGMP metabolism has already been observed in retinal rod outer segments, where the guanine-nucleotide-binding protein, transducin, is coupled to a light (rhodopsin) activated cGMP-specific phosphodiesterase [32,33].

It should be noted that in the case of the large, polymeric cholera and pertussis toxins, it is the free A-subunit of the toxin which is internalized and acts as the enzymatic activity responsible for ADP-ribosylating the G-proteins [27]. STa, on the other hand, is a small (1900 Da) peptide toxin that has no endogenous ADP-ribosyltransferase activity, and is not thought to be internalized during the early stages of its action (unpublished). Thus, if STa does lead directly to ADP-ribosylation events, it may be doing so by binding to the STa receptor [21,22] and then triggering the activation of cellular ADP-ribosyltransferases, which have now been described in a number of cell types [34–36]. Subsequent ADP-ribosylation of G-proteins might then act to alter guanylate cyclase and/or cGMP phosphodiesterase directly, or might act first on a sensitive phospholipase activity as has been recently suggested [37,38]. Such activation of phospholipase A activity would liberate free fatty acids and their oxidative metabolites, which are known to be potent activators of guanylate cyclase in many tissues [39,40], while the effects of phospholipase C activation on guanylate cyclase or cGMP phosphodiesterase remain unknown.

Further studies are clearly needed to elucidate fully the mechanism of STa-induced intestinal secretion. In particular, it is not clear at present why PT and NAD cause opposite effects on cGMP accumulation. However, we have recently observed that rat intestine contains at least 3 G-proteins (unpublished) and it is possible that we are seeing the result of 2 distinct, yet related events. Nonetheless, the findings reported here do suggest

that at least in the intestine, there may be some analogies between the G-protein/cAMP system and the less understood cGMP system. In wanting to validate further and study these analogies, future efforts will be directed towards identifying the specific intestinal G-proteins and observing how [$^{32}$P]ADP-ribosylation of these and other membrane proteins lead to alterations in intestinal cGMP metabolism.

## ACKNOWLEDGEMENTS

We thank Genetic Diagnostics Corporation for their generous and continuing support of this work. We also acknowledge the advice and interest of Dr Craig C. Malbon, and we thank Ms Francine Capolupo for preparation of the manuscript.

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